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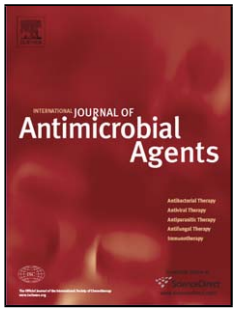

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Intrinsic fluoroquinolone resistance in *Orientia tsutsugamushi*

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ABSTRACT

Scrub typhus is a public health concern for a population of over a billion humans, with an estimated incidence of one million cases/year in endemic areas. Although doxycycline remains the standard therapy, fluoroquinolones have been used successfully in a few patients. However, there is also clinical evidence that fluoroquinolones are ineffective in the treatment of scrub typhus. To clarify this matter, we determined the in vitro susceptibility of *Orientia tsutsugamushi* strain Kato to ciprofloxacin and sequenced the quinolone resistance-determining region (QRDR) of the *gyrA* gene, the target of fluoroquinolones, of 18 fresh isolates from Lao PDR. *Orientia tsutsugamushi* strain Kato was resistant to ciprofloxacin and ofloxacin in vitro (minimum inhibitory concentration = 8 μg/mL). All sequences obtained, including those from the two available genomes of *O. tsutsugamushi* (strains Boryong and Ikeda), had a Ser83Leu mutation in their QRDR domain that is known to be associated with fluoroquinolone resistance. These findings re-emphasise the usefulness of in silico analysis for the prediction of antibiotic resistance and suggest that fluoroquinolones should not be used in the treatment of scrub typhus.

1. Introduction

Orientia tsutsugamushi, the agent of scrub typhus, is a small Gram-negative obligate intracellular bacterium of the alpha subgroup of Proteobacteria, transmitted to humans by the bite of trombiculid mites. The disease is a public health concern for a population of over a billion humans in a geographical triangle extending from northern Japan and far eastern Russia in the north, to northern Australia in the south and Pakistan and Afghanistan in the west [1–3]. The estimated incidence of the disease in endemic areas is one million cases/year. Although doxycycline and chloramphenicol remain the gold standard therapy for scrub typhus, ciprofloxacin has been shown to be effective in a mouse model [4] and in some anecdotal clinical cases [5–7]. However, recent reports of clinical failure and deaths as well as abortions in pregnant women with fluoroquinolone therapy suggest drug resistance in these cases [8–10]. Fluoroquinolone activity is due to inhibition of bacterial DNA gyrase (topoisomerase II) and topoisomerase IV. It has been demonstrated that resistance to quinolones in intracellular bacteria was mainly due to point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*) [11,12]. Surprisingly, there are only a few reports regarding in vitro and/or in vivo antibiotic susceptibility in cell culture or animal models of *O. tsutsugamushi*, especially for fluoroquinolones. In the in vitro work of Kelly et al. [13], although they determined precisely the doxycycline minimum inhibitory concentration (MIC) against *O. tsutsugamushi* Karp strain, they tested only one high concentration of ciprofloxacin (4 μg/mL) that was found to be effective in vitro. Similarly, McLain et al. [4] found that ciprofloxacin was effective in preventing death in a mouse model of scrub typhus, but the appropriateness of such a mouse model to human disease is uncertain and the

dose (mg/kg body weight) of ciprofloxacin administered to mice was approximately six times the adult human dosage. With a lack of definitive data regarding the susceptibility of *O. tsutsugamushi* to fluoroquinolones, the objectives of this study were: (i) to determine the MIC of ciprofloxacin and ofloxacin against *O. tsutsugamushi* strain Kato using a modified real-time quantitative polymerase chain reaction (qPCR) assay previously used for *Rickettsia* spp. and *Coxiella burnetii* [14,15]; (ii) to amplify and sequence the *gyrA* gene of recent human *O. tsutsugamushi* isolates from Lao PDR (Laos); and (iii) to compare these sequences with those retrieved from available full sequenced genomes of strains Boryong [16] and Ikeda [17] in order to decipher the possible molecular mechanism of resistance to fluoroquinolones.

2. Materials and methods

2.1. Patients and rickettsial culture

Patients were recruited to a study of the causes of fever [1]. Buffy coats, prepared from 5 mL of whole ethylene diamine tetra-acetic acid (EDTA)-anticoagulated venous blood from patients with suspected scrub typhus and serum immunoglobulin M (IgM) against *O. tsutsugamushi* detected by the scrub typhus immunochromatographic test (PanBio, Sinnamon Park, QLD, Australia), were cultured in Vero cells and L929 cells at 35 °C in 10% fetal calf serum/RPMI in a 5% CO_2 incubator [18]. DNA was extracted using High Pure PCR Template Preparation Kit (Roche Diagnostics, Meylan, France) and sent to URMITE (Marseille, France).

2.2. Preparation of Orientia tsutsugamushi inocula

Orientia tsutsugamushi strain Kato (CSUR R163) was propagated in monolayers of L929 cells grown in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine (Invitrogen, Cergy-Pontoise, France). When almost 100% of the floating cells were infected, as determined by Giemsa (Merck, Darmstadt, Germany) staining, cells were harvested, disrupted by glass beads (diameter 3 mm) and centrifuged at 500 × *g* for 5 min at 4 °C. The supernatant containing *O. tsutsugamushi* was centrifuged again at 2000 × *g* for 10 min at 4 °C to collect the bacterial pellet. *Orientia tsutsugamushi* was cryopreserved in MEM containing 20% FBS and 5% dimethyl sulphoxide (DMSO) and stored at –80 °C until use. The infectivity titre of inocula was determined as described previously [19] with slight modification. Briefly, the inoculum was five-fold serially diluted and inoculated onto L929 cells grown in a 24-well plate. After 2 h of inoculation, the inoculum was removed and replaced by new media containing 0.4 μg/mL daunorubicin (BIOMOL, Lausen, Switzerland), which partially inhibits the growth of host cells [20]. After 2 days of incubation, cells were collected and stained by indirect immunofluorescence assay using pooled human sera from Thai patients [2] with scrub typhus at a dilution of 1/400. Fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (FluolineG; bioMérieux, Marcy l'Etoile, France) diluted at 1/200 was used as the secondary antibody. The infected cell counting units (ICU) of *O. tsutsugamushi* were calculated from the dilution in the well in which 5–25% of cells were infected, using the formula ICU = total number of cells × percentage of infected cells/100 × dilution factor.

2.3. Antibiotic susceptibility testing

Growth of intracellular *O. tsutsugamushi* in cell culture with different concentrations of antibiotics was determined using real-time PCR with a TaqMan® probe as previously described for *Rickettsia* [15]. Briefly, 3×10^5 ICU of *O. tsutsugamushi* was inoculated onto monolayers of 1.5×10^5 L929 cells grown in 24-well plates. After incubation for 2 h, the inoculum was removed and infected cells were washed with phosphate-buffered saline. Infected cells were grown in media without antibiotic or with two-fold serial dilutions of antibiotics and were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell monolayers were harvested by trypsinisation and the cell suspension was centrifuged to collect cell pellets. Samples were collected every 24 h throughout the 5-day experiments. Experiments were repeated twice and each experiment was performed in duplicate. Cell pellets containing intracellular *O. tsutsugamushi* were processed for DNA extraction using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA copy number of *O. tsutsugamushi* was quantified using a real-time PCR targeting the *rpoB* gene. The sequence of the primers and the TaqMan probe were as follows: rpoB F (3235–3257), 5'-AAG CAT AGG TTA CAG CCT GGW GA-3'; rpoB R (3346–3373), 5'-ACC CCA ACG GAT TTA ATA CTA TAT CWA C-3'; and rpoB probe R (3307–3338), 5'-FAM-CCA TCT TCA AGA AAT GGC ATA TCT TCC TCA GG- TAMRA-3'. The real-time PCR mixture contained 1× QuantiTect Probe PCR Master Mix (Qiagen), 0.5 μM forward and reverse primers, 0.2 μM probe, sterile distilled water and 5 μL of DNA. Real-time PCR was performed in a Smart Cycler Instrument (Cepheid, Paris, France) with the following conditions: initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 5 s and combined

annealing and extension at 60 °C for 60 min with the acquisition of fluorescence. The number of DNA copies was calculated using the standard curve technique.

2.4. Determination of the quinolone resistance-determining region of gyrA and protein sequences alignment

Eighteen strains of *O. tsutsugamushi* isolated from the blood of Lao patients with scrub typhus [1] were used for determination of the QRDR sequence of *gyrA*. DNA was extracted from the blood sample (buffy coat layer) using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Primers used for partial *gyrA* amplification and sequencing were as follows: TsgyrAF, 5'-TATGCTATGAGCGTAATAGT-3'; and TsgyrAR, 5'-TGCCATTCCTACTGCAATTC-3'. Finally, the partial *gyrA* sequences of the QRDR of *O. tsutsugamushi* were aligned using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/) to look at possible mutations known to be associated with fluoroquinolone resistance. The *gyrA* protein sequences of *O. tsutsugamushi* strain Boryong (OTBS_0252) and Ikeda (OTT_1379) and *Escherichia coli* K12 (b2231) retrieved at the KEGG website (http://www.genome.jp/) were used for comparison of positions within the gene.

3. Results and discussion

In the real-time qPCR assay, the MIC determined in duplicate against ciprofloxacin and ofloxacin for *O. tsutsugamushi* strain Kato was 8 μg/mL (Fig. 1). Experiments were carried out twice to confirm the results. Compared with *E. coli*, the QRDR region of *gyrA* of the 18 Lao *O. tsutsugamushi* isolates as well as those retrieved

from the KEGG website (strains Boryong and Ikeda) displayed an intrinsic Ser83Leu mutation (Fig. 2). Moreover, 19 of the 20 sequences had a Thr88Ser mutation, 1 isolate had a Thr88Ala mutation and all 20 protein sequences had an Ile89Leu mutation. Thus, looking at in vitro and in silico results, it was found that *O. tsutsugamushi* strain Kato was intrinsically resistant to ciprofloxacin and that all available sequences of the *O. tsutsugamushi* QRDR domain of *gyrA* had an intrinsic mutation at position 83 known to be associated with fluoroquinolone resistance, as has been established in other intracellular bacteria including *Ehrlichia* spp., *Bartonella* spp. and *Tropheryma whipplei* [11,12]. Therefore, these data provide evidence that *O. tsutsugamushi* is naturally resistant to fluoroquinolones, explaining clinical failures reported using such antibiotics in the treatment of scrub typhus. Indeed, there are several reports demonstrating that ciprofloxacin is not effective in the treatment of scrub typhus (Table 1). In an outbreak in Southern India, among 28 patients serologically confirmed to have scrub typhus, 17 patients treated with doxycycline and 2 patients treated with chloramphenicol recovered in 1–3 days [9]. However, in five patients who received ciprofloxacin, fever subsided only after 5 days. Three patients (10.7%) died, including one patient treated with doxycycline and two with fluoroquinolones, indicating a possibility of drug resistance [9]. Similarly, we have reported five pregnant Indian patients with scrub typhus, four of whom were treated initially with ciprofloxacin [8]. Three women had stillbirths, one an abortion and one a low birth weight baby [8]. Scrub typhus has also been transmitted by needle stick from a patient who received pefloxacin [21]. Finally, Tsai et al. [10] have shown recently in a retrospective case series of 132 patients with scrub typhus that although treatment with levofloxacin was effective in 71 patients, the patients had a significantly longer time to defervescence compared with 61 patients treated with

tetracyclines. Moreover, for patients with severe scrub typhus, higher mortality was observed in the levofloxacin-treated group [10]. There are three reports of apparent successful scrub typhus therapy with fluoroquinolones [5,6,22], although the evidence for scrub typhus as the aetiology of the disease is uncertain for one patient [5]. Our in vitro and in silico findings re-emphasise the usefulness of such analysis for prediction of antibiotic resistance in intracellular bacteria. These results suggest that fluoroquinolones should not be used for the treatment of scrub typhus. Fluoroquinolones remain efficacious for the therapy of uncomplicated typhoid fever in much of Asia. Therefore, with the difficulty in clinically distinguishing typhus from typhoid, innate resistance in *O. tsutsugamushi* to fluoroquinolones has important practical implications for the empirical treatment of undifferentiated fever in Southeast Asia, suggesting that fluoroquinolones are unlikely to have sufficiently broad pathogen coverage.

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Competing interests

None declared.

Ethical approval

Ethical clearance was granted by the Faculty of Medical Sciences Ethical Review Committee, National University of Laos and the Oxford University Tropical Research Ethics Committee.

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Fig. 1. Growth curves of *Orientia tsutsugamushi* in L929 cells with different concentrations of (A) ciprofloxacin and (B) ofloxacin. The number of bacteria was expressed as log DNA copies. Experiments were repeated twice and each experiment was performed in duplicate. Data in the figure represent mean ± standard deviation.

Fig. 2. (A) DNA sequence alignment of the quinolone resistance-determining region (QRDR) domain of the *gyrA* genes of *Orientia tsutsugamushi* strains from Laos and from genomes of strains Boryong and Ikeda showing the Ser83Leu mutation (*Escherichia coli* numbering); and (B) three-dimensional representation of DNA gyrase showing position 83 in the QRDR.

Table 1

Epidemiological and clinical data on fluoroquinolone therapy for scrub typhus

Patient [age (years)]	Country	Antibiotic	Outcome	Reference
Female (32)	Thailand	Cefotaxime + ofloxacin	Cured	[5]
Male (34)	Sri Lanka	Doxycycline + ciprofloxacin	Cured	[7]
Male (20)	Korea	Pefloxacin + cefazolin	Clinical deterioration	[20]
Female (17) [a]	India	Ciprofloxacin	No improvement; stillbirth	[8]
Female (20) [a]	India	Ciprofloxacin	No improvement; stillbirth	[8]
Female (24) [a]	India	Ciprofloxacin + ampicillin	No improvement; low birth weight baby	[8]
Female (21) [a]	India	Ciprofloxacin	No improvement; abortion	[8]
Male and female patients (*n* = 5)	India	Ciprofloxacin	Longer time to defervescence	[9]
Male and female patients (*n* = 71)	Taiwan	Levofloxacin	Longer time to defervescence; 4 deaths	[10]

[a] Pregnant women.

Edited Figure 1

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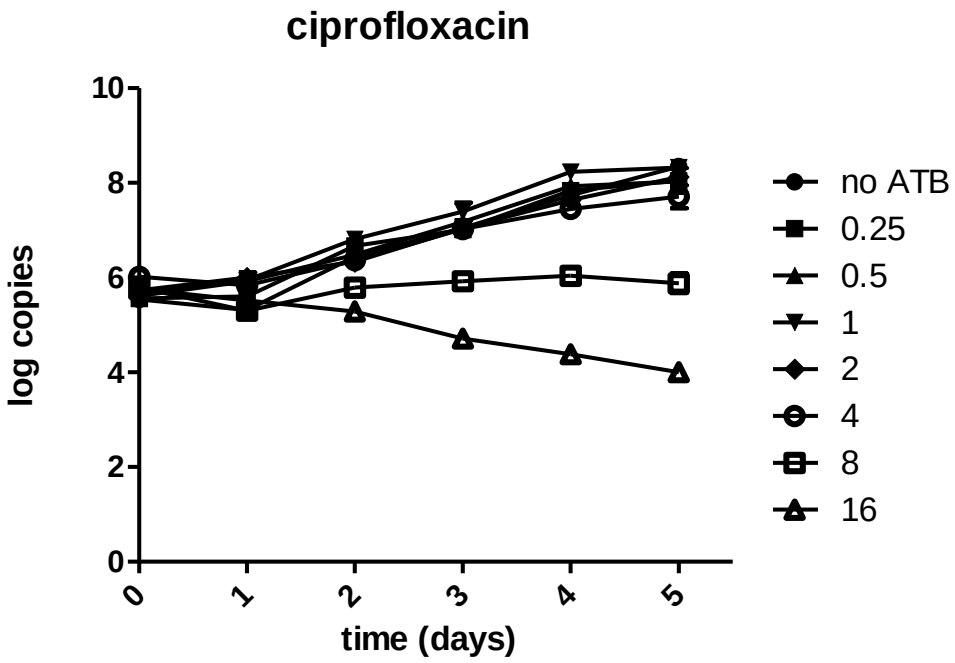

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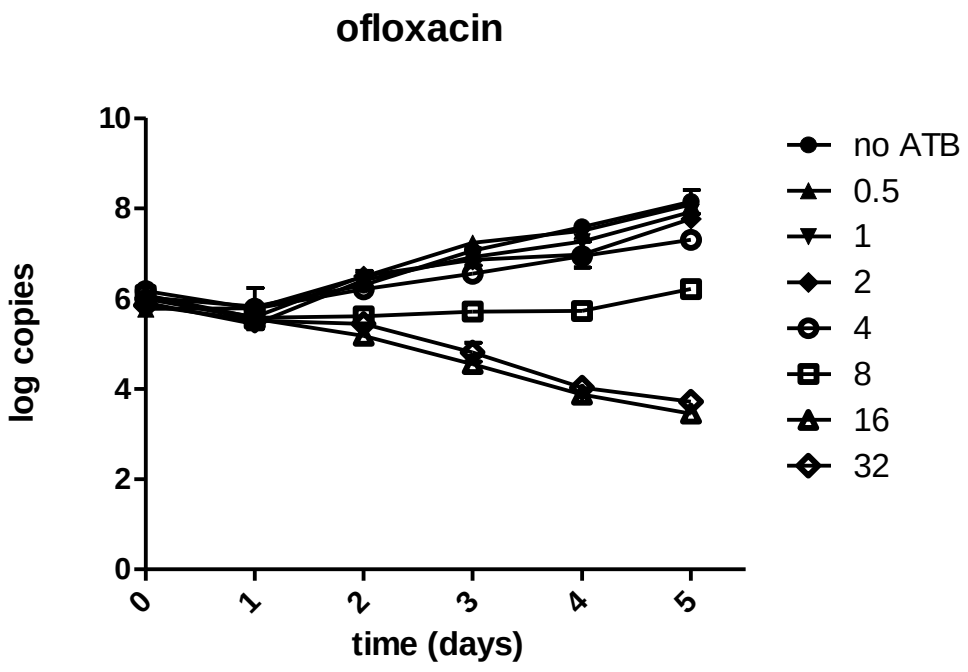

Edited Figure 2

A

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Kato          HYNKQYRKSARVVGDVIGKYHPHGELAVYDSLVRMAQDFSLRLPLVDGQGNFGSMDGDAA
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2325          HYNKQYRKSARVVGDVIGKYHPHGELAVYDSLVRMAQDFSLRLPLVDGQGNFGSMDGDAA
2223          HYNKQYRKSARVVGDVIGKYHPHGELAVYDSLVRMAQDFSLRLPLVDGQGNFGSMDGDAA
2261          HYNKQYRKSARVVGDVIGKYHPHGELAVYDSLVRMAQDFSLRLPLVDGQGNFGSMDGDAA
Boryong       HYNKQYRKSARVVGDVIGKYHPHGELAVYDSLVRMAQDFSLRLPLVDGQGNFGSMDGDAA
2259          HYNKQYRKSARVVGDVIGKYHPHGELAVYDSLVRMAQDFSLRLPLVDGQGNFGSMDGDAA
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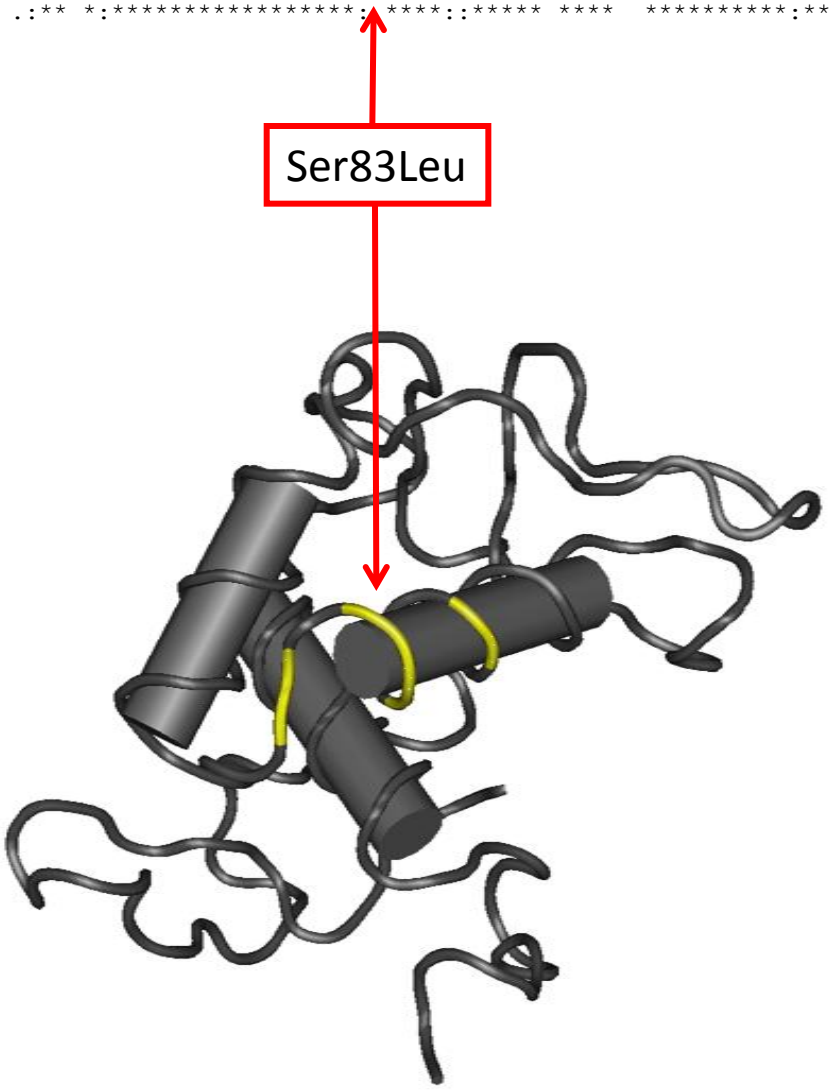

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